Materials and Methods for Treatment of Allergic Disease

Field of the Invention

The present invention relates to materials and methods for the treatment of allergic disease, and particularly although not exclusively, to nucleic acids for use in repressing the expression of cellular STAT6 ribonucleic acid, peptide, polypeptide or protein.

Background to the Invention

The incidence and cost of treating respiratory tract allergic disease is increasing. Cost-efficient, more effective, or preventative therapeutics are therefore desirable[1].

One such allergic disease is asthma in which the inflammatory pathology is predominantly mediated by cytokines which utilise a common intra-cellular transcription factor known as STAT6 (signal transducer and activator of transcription 6). STAT6 is critical for allergy development, mucosal/ airway inflammation and asthma (STAT6-deficient animals do not get asthma, even when challenged in a way that induces asthma in normal mice).

Drugs that specifically and effectively target STAT6, which resides and operates in the intracellular environment, have proved difficult to develop. For example, anti-STAT6 peptides have been investigated[13] but were found to achieve only limited and very transient (minutes) repression of STAT6 protein expression. The

transient effect is considered to be due to peptide degradation by endogenous cellular proteases.

Attempts to repress STAT6 expression in vivo through antisense DNA techniques[15] have proved unsuccessful. This approach suffers from a series of problems. For example only a low inhibition of STAT6 expression is obtained, even at high concentrations of antisense DNA, the effects are transient and the antisense molecule is subject to degradation and is difficult to target to the appropriate intracellular location. The high concentration of antisense DNA required to produce any useful effect often causes the antisense DNA to exhibit antigenic properties and can invoke an immune response. Furthermore, mice treated with STAT6 directed antisense DNA did not exhibit an improvement in allergic symptoms and developed splenomegaly[16], i.e. a toxic side effect.

Accordingly, to date, STAT6 has proved to be a very difficult molecule to effectively inhibit or repress in a therapeutically useful manner. Despite several attempts, no successful drug or composition has been developed that targets STAT6 effectively without causing non-specific side-effects.

STAT6

STAT6 is the Signal Transducer and Activator of Transcription 6. To be functional in intact cells, STAT6 has to be phosphorylated. Sequence data for human STAT6 can be accessed from NCBI (www.ncbi.nlm.nih.gov) under accession numbers NP_003144 (NM_003153) and U16031.

RNA interference (RNAi)

RNAi utilises small double-stranded RNA molecules (dsRNA) to target messenger RNA (mRNA), the precursor molecule that cells use to translate the genetic code into functional proteins. During the natural process of RNAi, dsRNA is enzymatically processed into short-interfering RNA (siRNA) duplexes of 21 nucleotides in length. The antisense strand of the siRNA duplex is then incorporated into a cytoplasmic complex of proteins (RNA-induced silencing complex or RISC). The RISC complex containing the antisense siRNA strand also binds mRNA which has a sequence complementary to the antisense strand - allowing complementary base-pairing between the antisense siRNA strand and the sense mRNA molecule. The mRNA molecule is then specifically cleaved by an enzyme (RNase) associated with the RISC resulting in specific gene silencing[3,4]. For gene silencing (i.e. mRNA cleavage) to occur, anti-sense RNA (i.e. siRNA) has to become incorporated into the RISC. This is a natural and highly efficient process that occurs in all nucleated cells and whose origin is thought to be in mediating protection from transposable elements (e.g. viruses) and in normal regulation of gene expression. It is therefore distinct from the artificial process of introducing anti-sense DNA molecules into cells, where targeting of mRNA occurs through simple base-pairing of the naked anti-sense DNA molecule to its RNA target.

The advantages of RNAi over other gene-targeting strategies such as DNA anti-sense oligonucleotides can include its relative specificity, its enhanced efficacy, and the fact that siRNA treatment feeds into a natural RNAi pathway that is inherent to all cells.

However, the success of RNAi in gene repression or silencing is unpredictable, indeed the outcome can be highly variable and may depend on a variety of factors which include the accessibility of the genetic target (i.e. mRNA) and the efficiency of RNAi in the cell type being targeted.

Summary of the Invention

The inventors have designed and *in vitro* tested STAT6 siRNA (short interfering RNA). Despite the intrinsic unpredictability of the efficacy of this approach they obtained specific and highly efficient inhibition of the expression of STAT6 mRNA and protein in cell types found in lung tissue, indicating that these molecules will provide effective and specific targeting of STAT6 *in vivo*.

The evidence presented herein demonstrates that STAT6 siRNA, when transferred into cells by cationic lipid-mediated transfer, are indeed functional and efficiently inhibit STAT6 mRNA, and protein expression without obvious side-effects in human cells.

By targeting these siRNA to representative cells from human airways, the inventors have provided the basis of a new therapeutic treatment for allergic disease of the respiratory tract such as rhinitis and asthma. Non-atopic asthma may also be amenable to STAT6 siRNA therapy. In particular, STAT6 siRNA's may be used to treat the local cells of the respiratory tract via delivery systems such as liposomes or in aerosol form by a standard nebuliser device.

The siRNA's provided specifically and efficiently target STAT6 in that they reduce STAT6 gene expression by >90%. Furthermore, cells treated with STAT6-specific siRNA do not express detectable STAT6 protein expression and they do not exhibit phosphorylation of STAT6 protein in response to physiological stimulus with interleukin-4 - in other words, cells treated with individual STAT6 siRNA lose their ability to signal through an intracellular pathway that is heavily implicated in the development of allergic immune responses and associated diseases of the respiratory tract that include asthma and allergic rhinitis.

The inventors have also demonstrated that STAT6 targeted siRNA provide persistent inhibition of STAT6 expression in lung cells at low (nanomolar, nM) concentrations of siRNA. Furthermore, inhibition of functional STAT6 protein is achieved without induction of an interferon response. This response is often seen when long (>30 bp) double stranded RNA is introduced into mammalian cells - the interferon response occurs naturally in response to viruses which harbour dsRNA, resulting in non-specific suppression of cellular gene expression. The fact that no such response is seen in the target cell group is further indicative of the efficacy of the siRNA approach taken by the inventors.

The inventors have demonstrated that STAT6 targeted siRNA provide potent, non-toxic, inhibitors of STAT6 function at very low concentration.

Treatment of allergic inflammation of the respiratory tract may be achieved by taking advantage of nebulisers or nasal sprays to deliver STAT6 siRNA. These delivery

methods are already standard in conventional treatments. Furthermore, for asthma or rhinitis, delivery of siRNA may take advantage of available commercial formulations (e.g. Smarticles®, Novosom AG, Germany)in aerosol or liquid form.

At its most general the present invention relates to nucleic acids, particularly siRNA, and their uses in repressing or silencing the expression of nucleic acids, peptides, polypeptides or protein.

More particularly, the present invention relates to the repression of STAT 6 ribonucleic acid (e.g. mRNA), peptide, polypeptide or protein expression. Ribonucleic acids, particularly in the form of siRNA, are provided having substantial sequence identity or complementarity along their length to all or a portion or fragment of at least one RNA sequence coding for a STAT6 protein. Such RNA sequences may include an RNA sequence (e.g. mRNA) encoding a STAT6 protein (e.g. the protein encoded by one of amino acid sequences SEQ ID No.s 9, 11 or 13) or one of the RNA sequences encoded by one of SEQ ID No.s 10, 12 or 14.

The use of such ribonucleic acids (siRNA) in the treatment of respiratory tract allergic or non-allergic disease, e.g. asthma or rhinitis, and in the manufacture of a medicament for the treatment of respiratory tract allergic or non-allergic diseases together with methods of treating respiratory tract allergic or non-allergic diseases are also provided.

The inventors have also provided methods of repressing or silencing the expression of a STAT6 ribonucleic acid

(mRNA) or protein in vitro and cells in which STAT6 ribonucleic acid or protein expression is repressed and which may be obtainable by such methods.

In one aspect of the invention a ribonucleic acid, particularly double stranded siRNA, is provided for use in the treatment of respiratory tract allergic disease in an individual.

The ribonucleic acid (siRNA) preferably represses the expression of STAT6 ribonucleic acid (mRNA), polypeptide or protein. Preferably STAT6 ribonucleic acid (mRNA) or protein function is also repressed.

Nucleic acids according to the invention may be DNA or RNA and may be single or double stranded. Preferably the nucleic acid is an RNA and is double stranded.

Preferred nucleic acids include RNA molecules having a sequence of, or complementary to, any of SEQ ID No.s 1-8 and nucleic acids having a sequence identity of at least 60% to one of SEQ ID No.s 1-8 or a complementary sequence thereof, and more preferably having at least 70, 80, 85, 90, 95% or 100% sequence identity. DNA molecules encoding RNA's comprising these sequences are also provided.

Isolated nucleic acids which may include an RNA molecule having a sequence of, or complementary to, any of SEQ ID No.s 1-8, nucleic acids having a sequence identity of at least 60% to one of SEQ ID No.s 1-8 or a complementary sequence thereof, and more preferably having at least 70, 80, 85, 90, 95 or 100% sequence identity, and DNA

molecules encoding RNA's comprising these sequences form another aspect of the invention.

In a further aspect of the invention the nucleic acids described, e.g. double stranded siRNA, are provided for use in the manufacture of a medicament for the treatment of respiratory tract allergic or non-allergic disease, e.g. asthma or rhinitis. Preferably the mechanism of treatment comprises the repression of expression of a STAT6 ribonucleic acid (mRNA) and/or protein in vivo.

In yet a further aspect of the invention a method of treating respiratory tract allergic disease in an individual in need of such treatment is provided. The method may comprise the step of administering to the individual an amount of one or more of the ribonucleic acids described herein, e.g. siRNA, which is effective to treat the symptoms of these disorders.

The individual to be treated may be a patient in need of treatment. The patient may be any animal or human. The patient may be a non-human mammal (e.g. mouse, rat or other mammal from the order Rodentia), but is more preferably a human patient. The patient may be male or female.

Medicaments and pharmaceutical compositions according to aspects of the present invention may be formulated for administration by a number of routes, including but not limited to, parenteral, intravenous, intramuscular, intratumoural, oral, oral inhalation and nasal. The medicaments and compositions may be formulated in fluid or solid form. Fluid formulations may be formulated for administration by injection to a selected region of the

human or animal body. Fluid formulations may be provided which are capable of being administered by aerosol.

In another aspect of the invention, a method is provided for repressing or silencing the cellular expression of STAT6 ribonucleic acid (mRNA)or protein in vitro. The method may comprise the contacting of a cell or cells with a nucleic acid described herein, e.g. a ribonucleic acid such as an siRNA, to deliver the nucleic acid (e.g. siRNA) to the cell(s). In one arrangement the nucleic acid (e.g. siRNA) may be complexed with a carrier, e.g. a lipophilic carrier to assist and/or enhance passage of the nucleic acid across the cell membrane.

Accordingly, cells may be provided in which the expression of STAT6 ribonucleic acid (mRNA)or protein is repressed or silenced.

Suitable cells may be selected from human cells, or alternatively from non-human cells, preferably rat, mouse or other rodent (including cells from any animal in the order Rodentia). Other suitable non-human cells may be e.g., from pig, sheep, non-human primate or other non-human vertebrate organism and/or non-human mammalian cells.

Ribonucleic acids of the invention may be prepared as part of a pharmaceutical composition comprising a carrier, e.g. a lipophilic carrier or vesicle, or adjuvant in addition to the nucleic acid. Pharmaceutical compositions and medicaments of the invention may be formulated for oral inhalation or nasal administration, alternatively for parenteral, intravenous or intramuscular administration.

For the treatment of repiratory tract allergy, suitable medicaments or therapeutics include those suitable for nasal and/or oral administration (preferably by inhalation) and may be provided as a solution suitable for generation of aerosolised droplets of the medicament for delivery to the airways and lungs by use of an appropriate nebuliser or inhaler. Compositions and medicaments according to the present invention may be formulated for delivery to the respiratory tract, e.g. intranasally, inhalationally or orally. Such compositions and medicaments may comprise suitable siRNA molecules with or without a suitable transfection reagent[9].

A number of transfection reagents suitable for in vivo delivery of siRNA molecules are known to the person skilled in the art[10]. Examples of such agents include lipophilic agents such as liposomes or commercially available agents such as Neophectin™ (Neopharm) and Smarticles® (Novosom AG). Pulmonary surfactant may also be used to deliver siRNA into the lungs. Pulmonary surfactants are commercially available (e.g. CuroSurf™) and are advantageous in that they are already clinically established and validated for safe use in humans.

According to one aspect of the present invention there is provided an isolated double stranded short interfering ribonucleic acid (siRNA) molecule that represses or silences expression of STAT6 nucleic acid (e.g. a STAT6 ribonucleic acid such as mRNA) or protein.

The sense strand of the siRNA may comprises a contiguous nucleotide sequence, wherein the base sequence of the

sense strand has at least 70% sequence identity to the base sequence of a contiguous nucleotide sequence of corresponding length which is contained in (i.e. is embedded within, or is a part, all or a fragment of) the mRNA sequence encoded by one of the human, mouse or rat STAT6 nucleotide sequences (SEQ ID No.s 10, 12 or 14). The contiguous nucleotide sequence of corresponding length contained in the mRNA sequence may be the RNA sequence of any one of SEQ ID No.s 5-8 or the RNA sequence encoded by any one of SEQ ID No.s 15-18.

The antisense strand of the siRNA may comprise a contiguous nucleotide sequence, wherein the base sequence of the antisense strand has at least 70% sequence complementarity to the base sequence of a contiguous nucleotide sequence of corresponding length which is contained in (i.e. is embedded within, or is a part, all or a fragment of) the mRNA sequence encoded by one of the human, mouse or rat STAT6 nucleotide sequences (SEQ ID No.s 10, 12 or 14). The contiguous nucleotide sequence of corresponding length contained in the mRNA sequence may be the RNA sequence of any one of SEQ ID No.s 5-8 or the RNA sequence encoded by any one of SEQ ID No.s 15-18.

The specified degree of sequence identity or complementarity may be at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%.

The anti-sense strand may be entirely complementary to said sense strand. The sense and antisense strands may be of the same length or of different lengths. Each strand may have a length in the range of 10 to 30 nucleotides, 15 to 25 nucleotides or 18 to 23

nucleotides. More preferably, each strand may have a length which is one of 19, 20, 21 or 22 nucleotides.

siRNA according to the present invention may have an antisense strand that hybridises to the mRNA encoded by one of SEQ ID No.s 10, 12 or 14 under high or very high stringency conditions.

Similarly, siRNA according to the present invention may have a sense strand that hybridises to one of SEQ ID No.s 10, 12 or 14 under high or very high stringency conditions.

In one arrangement, the sense or antisense strand may hybridise to the corresponding other strand of one of SEQ ID No.s 1-4 under high or very high stringency conditions.

In another arrangement the siRNA may have a sequence identity of at least 70% to the corresponding strand of any one of SEQ ID No.s 1-4.

In yet another arrangement the antisense strand may have at least 70% sequence complementarity over the entire length of said siRNA to a portion or fragment of RNA sequence coding for a STAT6 protein. In yet a further arrangement the sense strand may have at least 70% sequence identity over the entire length of said siRNA to a portion or fragment of a STAT6 mRNA. The RNA (or mRNA) sequence may be that encoded by any one of SEQ ID No.s 10, 12 or 14.

siRNA according to the present invention may comprise, or consist of, any one of SEQ ID No.s 1, 2, 3 or 4.

Preferred siRNA act to repress the function and/or expression of STAT6 mRNA and/or STAT6 protein. siRNA according to the present invention may be provided for use in the treatment of respiratory tract allergic or non-allergic disease. Pharmaceutical compositions comprising siRNA according to the present invention are also provided. Suitable pharmaceutical compositions may be formulated for oral or nasal administration and may comprise a pharmaceutically acceptable diluent, carrier or adjuvant. One type of suitable carrier is a lipophilic carrier or vesicle.

In a further aspect of the present invention siRNA according to the present invention are provided for use in the manufacture of a medicament for the treatment of respiratory tract allergic disease or in non-atopic asthma. The medicament may be formulated for oral or nasal administration.

In yet a further aspect of the present invention a method of treating allergic or non-allergic disease in a patient in need of such treatment is provided, the method comprising the steps of administering to the patient an siRNA or pharmaceutical composition according to the present invention. Suitable pharmaceutical compositions may be formulated for oral or nasal administration.

The siRNA, pharmaceutical compositions, uses and methods of treatment forming part of the present invention may be useful in treating respiratory tract allergic or non-allergic disease. That allergic disease may be asthma or rhinitis. Non-allergic diseases may include non-atopic asthma.

In yet a further aspect of the present invention there is provided a method for repressing the cellular expression of STAT6 protein in vitro comprising, in vitro, contacting a cell with an siRNA according to the present invention.

In yet another aspect of the present invention there is provided a cell, in vitro, in which STAT6 protein or ribonucleic acid expression or function is repressed or silenced. The cell preferably comprises an siRNA according to the present invention.

Suitable cells may comprise mammalian cells (including non-human mammalian cells) or human cells and may be cells from the respiratory tract or the progeny of cells from the respiratory tract, e.g. human bronchial epithelial cells. Components of the respiratory tract may include the trachea, lungs, bronchi or alveoli.

Nucleic acids of the invention may include any of the following double or single stranded RNA sequences.

	Sequence ID No.
5′-GCAGGAAGAACUCAAGUUUtt-3′ 3′-ttCGUCCUUCUUGAGUUCAAA-5′	1
5′-ACAGUACGUUACUAGCCUUtt-3′ 3′-ttUGUCAUGCAAUGAUCGGAA-5′	2
5′-GAAUCAGUCAACGUGUUGUtt-3′ 3′-ttCUUAGUCAGUUGCACAACA-5	3
5′-AGCACUGGAGAAAUCAUCAtt-3′	4

3′-ttUCGUGACCUCUUUAGUAGU-5′

GCAGGAAGAACUCAAGUUU	5
ACAGUACGUUACUAGCCUU	6
GAAUCAGUCAACGUGUUGU	7
AGCACUGGAGAAAUCAUCA	8

Furthermore, ribonucleic acids of the invention may comprise ribonucleic acid molecules which hybridise with any of SEQ ID No.s 1 to 8 under very high, high or intermediate stringency conditions.

siRNA molecules of the present invention may be designed using the sequence information for STAT6 ribonucleic acid and protein that is available in the art. Figures 4 to 6 provide nucleotide sequence information for the STAT6 gene of human, mouse and rat. From such a nucleotide sequence it is possible to design an siRNA molecule which specifically targets the expression and/or function of STAT6. For example, one can design and synthesise an siRNA molecule which has an antisense strand composed of a sequence of nucleotides complementary to a fragment of an RNA encoded by one of SEQ ID No.′s 10, 12 or 14, which RNA fragment may be encoded by a corresponding DNA fragment starting at any selected nucleotide of any one of SEQ ID No.s 10, 12 or 14. The complementary sense strand can also be readily designed and synthesised in order to provide a double stranded STAT6 siRNA.

siRNA molecules of the invention may be designed to optionally incorporate, as part of the siRNA, two contiguous thymine bases at the 3′ end of one or each strand of the siRNA molecule. These thymine bases

preferably "overhang" the 5' end of the opposing strand. These thymine bases are preferably part of (or are encoded by) the natural DNA or RNA sequence which the sense or antisense strand of the siRNA is based on. Alternatively they may be deliberately incorporated during synthesis of the siRNA.

siRNA molecules of the invention may be of any length, but preferred nucleic acids are small and may have a strand length of at least 10 nucleotides and no more than 50 nucleotides. Particularly suitable siRNA will have a strand length in the range 10 to 30 nucleotides and more suitably in the range 15 to 25 nucleotides. Selected siRNA may have a strand length of any one of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides in length. For a double stranded siRNA having a strand length of, say, 21 nucleotides, this means that each strand of the duplex is 21 nucleotides in length. Whilst it may be preferred that each strand of a double stranded siRNA is of the same length, this is not essential and each strand may be of separate defined length.

Thus, a STAT6 specific siRNA molecule having a specified length selected in accordance with the above may be prepared having an antisense strand which has a specified degree of complementarity to a selected part or fragment of the RNA molecule encoded by any one of SEQ ID No.'s 10, 12 or 14. The antisense strand may have substantial sequence complementarity (i.e. at least 70%, more preferably one of 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100%) to a selected part or fragment of the RNA encoded by one of SEQ ID No's 10, 12 or 14, wherein that part or fragment has a length selected in accordance with this disclosure, and wherein that part or fragment has a

contiguous sequence of nucleotides encoded by a corresponding part or fragment of one of SEQ ID No.s 10, 12 or 14 and wherein the encoding part or fragment may start from any one of nucleotide positions:

1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342,

343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, 633, 634, 635, 636, 637, 638, 639, 640, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716,

717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851, 852, 853, 854, 855, 856, 857, 858, 859, 860, 861, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 873, 874, 875, 876, 877, 878, 879, 880, 881, 882, 883, 884, 885, 886, 887, 888, 889, 890, 891, 892, 893, 894, 895, 896, 897, 898, 899, 900, 901, 902, 903, 904, 905, 906, 907, 908, 909, 910, 911, 912, 913, 914, 915, 916, 917, 918, 919, 920, 921, 922, 923, 924, 925, 926, 927, 928, 929, 930, 931, 932, 933, 934, 935, 936, 937, 938, 939, 940, 941, 942, 943, 944, 945, 946, 947, 948, 949, 950, 951, 952, 953, 954, 955, 956, 957, 958, 959, 960, 961, 962, 963, 964, 965, 966, 967, 968, 969, 970, 971, 972, 973, 974, 975, 976, 977, 978, 979, 980, 981, 982, 983, 984, 985, 986, 987, 988, 989, 990, 991, 992, 993, 994, 995, 996, 997, 998, 999, 1000, 1001, 1002, 1003, 1004, 1005, 1006, 1007, 1008, 1009, 1010, 1011, 1012, 1013, 1014, 1015, 1016, 1017, 1018, 1019, 1020, 1021, 1022, 1023, 1024, 1025, 1026, 1027, 1028, 1029, 1030, 1031, 1032, 1033, 1034, 1035, 1036, 1037, 1038, 1039, 1040, 1041, 1042, 1043, 1044, 1045, 1046, 1047, 1048, 1049, 1050, 1051, 1052, 1053, 1054, 1055, 1056, 1057, 1058, 1059, 1060, 1061, 1062, 1063, 1064, 1065, 1066, 1067, 1068, 1069, 1070, 1071, 1072, 1073, 1074,

1075, 1076, 1077, 1078, 1079, 1080, 1081, 1082, 1083, 1084, 1085, 1086, 1087, 1088, 1089, 1090, 1091, 1092, 1093, 1094, 1095, 1096, 1097, 1098, 1099, 1100, 1101, 1102, 1103, 1104, 1105, 1106, 1107, 1108, 1109, 1110, 1111, 1112, 1113, 1114, 1115, 1116, 1117, 1118, 1119, 1120, 1121, 1122, 1123, 1124, 1125, 1126, 1127, 1128, 1129, 1130, 1131, 1132, 1133, 1134, 1135, 1136, 1137, 1138, 1139, 1140, 1141, 1142, 1143, 1144, 1145, 1146, 1147, 1148, 1149, 1150, 1151, 1152, 1153, 1154, 1155, 1156, 1157, 1158, 1159, 1160, 1161, 1162, 1163, 1164, 1165, 1166, 1167, 1168, 1169, 1170, 1171, 1172, 1173, 1174, 1175, 1176, 1177, 1178, 1179, 1180, 1181, 1182, 1183, 1184, 1185, 1186, 1187, 1188, 1189, 1190, 1191, 1192, 1193, 1194, 1195, 1196, 1197, 1198, 1199, 1200, 1201, 1202, 1203, 1204, 1205, 1206, 1207, 1208, 1209, 1210, 1211, 1212, 1213, 1214, 1215, 1216, 1217, 1218, 1219, 1220, 1221, 1222, 1223, 1224, 1225, 1226, 1227, 1228, 1229, 1230, 1231, 1232, 1233, 1234, 1235, 1236, 1237, 1238, 1239, 1240, 1241, 1242, 1243, 1244, 1245, 1246, 1247, 1248, 1249, 1250, 1251, 1252, 1253, 1254, 1255, 1256, 1257, 1258, 1259, 1260, 1261, 1262, 1263, 1264, 1265, 1266, 1267, 1268, 1269, 1270, 1271, 1272, 1273, 1274, 1275, 1276, 1277, 1278, 1279, 1280, 1281, 1282, 1283, 1284, 1285, 1286, 1287, 1288, 1289, 1290, 1291, 1292, 1293, 1294, 1295, 1296, 1297, 1298, 1299, 1300, 1301, 1302, 1303, 1304, 1305, 1306, 1307, 1308, 1309, 1310, 1311, 1312, 1313, 1314, 1315, 1316, 1317, 1318, 1319, 1320, 1321, 1322, 1323, 1324, 1325, 1326, 1327, 1328, 1329, 1330, 1331, 1332, 1333, 1334, 1335, 1336, 1337, 1338, 1339, 1340, 1341, 1342, 1343, 1344, 1345, 1346, 1347, 1348, 1349, 1350, 1351, 1352, 1353, 1354, 1355, 1356, 1357, 1358, 1359, 1360, 1361, 1362, 1363, 1364, 1365, 1366, 1367, 1368, 1369, 1370, 1371, 1372, 1373, 1374, 1375, 1376, 1377, 1378, 1379, 1380,

1381, 1382, 1383, 1384, 1385, 1386, 1387, 1388, 1389, 1390, 1391, 1392, 1393, 1394, 1395, 1396, 1397, 1398, 1399, 1400, 1401, 1402, 1403, 1404, 1405, 1406, 1407, 1408, 1409, 1410, 1411, 1412, 1413, 1414, 1415, 1416, 1417, 1418, 1419, 1420, 1421, 1422, 1423, 1424, 1425, 1426, 1427, 1428, 1429, 1430, 1431, 1432, 1433, 1434, 1435, 1436, 1437, 1438, 1439, 1440, 1441, 1442, 1443, 1444, 1445, 1446, 1447, 1448, 1449, 1450, 1451, 1452, 1453, 1454, 1455, 1456, 1457, 1458, 1459, 1460, 1461, 1462, 1463, 1464, 1465, 1466, 1467, 1468, 1469, 1470, 1471, 1472, 1473, 1474, 1475, 1476, 1477, 1478, 1479, 1480, 1481, 1482, 1483, 1484, 1485, 1486, 1487, 1488, 1489, 1490, 1491, 1492, 1493, 1494, 1495, 1496, 1497, 1498, 1499, 1500, 1501, 1502, 1503, 1504, 1505, 1506, 1507, 1508, 1509, 1510, 1511, 1512, 1513, 1514, 1515, 1516, 1517, 1518, 1519, 1520, 1521, 1522, 1523, 1524, 1525, 1526, 1527, 1528, 1529, 1530, 1531, 1532, 1533, 1534, 1535, 1536, 1537, 1538, 1539, 1540, 1541, 1542, 1543, 1544, 1545, 1546, 1547, 1548, 1549, 1550, 1551, 1552, 1553, 1554, 1555, 1556, 1557, 1558, 1559, 1560, 1561, 1562, 1563, 1564, 1565, 1566, 1567, 1568, 1569, 1570, 1571, 1572, 1573, 1574, 1575, 1576, 1577, 1578, 1579, 1580, 1581, 1582, 1583, 1584, 1585, 1586, 1587, 1588, 1589, 1590, 1591, 1592, 1593, 1594, 1595, 1596, 1597, 1598, 1599, 1600, 1601, 1602, 1603, 1604, 1605, 1606, 1607, 1608, 1609, 1610, 1611, 1612, 1613, 1614, 1615, 1616, 1617, 1618, 1619, 1620, 1621, 1622, 1623, 1624, 1625, 1626, 1627, 1628, 1629, 1630, 1631, 1632, 1633, 1634, 1635, 1636, 1637, 1638, 1639, 1640, 1641, 1642, 1643, 1644, 1645, 1646, 1647, 1648, 1649, 1650, 1651, 1652, 1653, 1654, 1655, 1656, 1657, 1658, 1659, 1660, 1661, 1662, 1663, 1664, 1665, 1666, 1667, 1668, 1669, 1670, 1671, 1672, 1673, 1674, 1675, 1676, 1677, 1678, 1679, 1680, 1681, 1682, 1683, 1684, 1685, 1686,

1687, 1688, 1689, 1690, 1691, 1692, 1693, 1694, 1695, 1696, 1697, 1698, 1699, 1700, 1701, 1702, 1703, 1704, 1705, 1706, 1707, 1708, 1709, 1710, 1711, 1712, 1713, 1714, 1715, 1716, 1717, 1718, 1719, 1720, 1721, 1722, 1723, 1724, 1725, 1726, 1727, 1728, 1729, 1730, 1731, 1732, 1733, 1734, 1735, 1736, 1737, 1738, 1739, 1740, 1741, 1742, 1743, 1744, 1745, 1746, 1747, 1748, 1749, 1750, 1751, 1752, 1753, 1754, 1755, 1756, 1757, 1758, 1759, 1760, 1761, 1762, 1763, 1764, 1765, 1766, 1767, 1768, 1769, 1770, 1771, 1772, 1773, 1774, 1775, 1776, 1777, 1778, 1779, 1780, 1781, 1782, 1783, 1784, 1785, 1786, 1787, 1788, 1789, 1790, 1791, 1792, 1793, 1794, 1795, 1796, 1797, 1798, 1799, 1800, 1801, 1802, 1803, 1804, 1805, 1806, 1807, 1808, 1809, 1810, 1811, 1812, 1813, 1814, 1815, 1816, 1817, 1818, 1819, 1820, 1821, 1822, 1823, 1824, 1825, 1826, 1827, 1828, 1829, 1830, 1831, 1832, 1833, 1834, 1835, 1836, 1837, 1838, 1839, 1840, 1841, 1842, 1843, 1844, 1845, 1846, 1847, 1848, 1849, 1850, 1851, 1852, 1853, 1854, 1855, 1856, 1857, 1858, 1859, 1860, 1861, 1862, 1863, 1864, 1865, 1866, 1867, 1868, 1869, 1870, 1871, 1872, 1873, 1874, 1875, 1876, 1877, 1878, 1879, 1880, 1881, 1882, 1883, 1884, 1885, 1886, 1887, 1888, 1889, 1890, 1891, 1892, 1893, 1894, 1895, 1896, 1897, 1898, 1899, 1900, 1901, 1902, 1903, 1904, 1905, 1906, 1907, 1908, 1909, 1910, 1911, 1912, 1913, 1914, 1915, 1916, 1917, 1918, 1919, 1920, 1921, 1922, 1923, 1924, 1925, 1926, 1927, 1928, 1929, 1930, 1931, 1932, 1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941, 1942, 1943, 1944, 1945, 1946, 1947, 1948, 1949, 1950, 1951, 1952, 1953, 1954, 1955, 1956, 1957, 1958, 1959, 1960, 1961, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992,

1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298,

2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 2544, 2545, 2546, 2547, 2548, 2549, 2550, 2551, 2552, 2553, 2554, 2555, 2556, 2557, 2558, 2559, 2560, 2561, 2562, 2563, 2564, 2565, 2566, 2567, 2568, 2569, 2570, 2571, 2572, 2573, 2574, 2575, 2576, 2577, 2578, 2579, 2580, 2581, 2582, 2583, 2584, 2585, 2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604,

2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 2679, 2680, 2681, 2682, 2683, 2684, 2685, 2686, 2687, 2688, 2689, 2690, 2691, 2692, 2693, 2694, 2695, 2696, 2697, 2698, 2699, 2700, 2701, 2702, 2703, 2704, 2705, 2706, 2707, 2708, 2709, 2710, 2711, 2712, 2713, 2714, 2715, 2716, 2717, 2718, 2719, 2720, 2721, 2722, 2723, 2724, 2725, 2726, 2727, 2728, 2729, 2730, 2731, 2732, 2733, 2734, 2735, 2736, 2737, 2738, 2739, 2740, 2741, 2742, 2743, 2744, 2745, 2746, 2747, 2748, 2749, 2750, 2751, 2752, 2753, 2754, 2755, 2756, 2757, 2758, 2759, 2760, 2761, 2762, 2763, 2764, 2765, 2766, 2767, 2768, 2769, 2770, 2771, 2772, 2773, 2774, 2775, 2776, 2777, 2778, 2779, 2780, 2781, 2782, 2783, 2784, 2785, 2786, 2787, 2788, 2789, 2790, 2791, 2792, 2793, 2794, 2795, 2796, 2797, 2798, 2799, 2800, 2801, 2802, 2803, 2804, 2805, 2806, 2807, 2808, 2809, 2810, 2811, 2812, 2813, 2814, 2815, 2816, 2817, 2818, 2819, 2820, 2821, 2822, 2823, 2824, 2825, 2826, 2827, 2828, 2829, 2830, 2831, 2832, 2833, 2834, 2835, 2836, 2837, 2838, 2839, 2840, 2841, 2842, 2843, 2844, 2845, 2846, 2847, 2848, 2849, 2850, 2851, 2852, 2853, 2854, 2855, 2856, 2857, 2858, 2859, 2860, 2861, 2862, 2863, 2864, 2865, 2866, 2867, 2868, 2869, 2870, 2871, 2872, 2873, 2874, 2875, 2876, 2877, 2878, 2879, 2880, 2881, 2882, 2883, 2884, 2885, 2886, 2887, 2888, 2889, 2890, 2891, 2892, 2893, 2894, 2895, 2896, 2897, 2898, 2899, 2900, 2901, 2902, 2903, 2904, 2905, 2906, 2907, 2908, 2909, 2910,

2911, 2912, 2913, 2914, 2915, 2916, 2917, 2918, 2919, 2920, 2921, 2922, 2923, 2924, 2925, 2926, 2927, 2928, 2929, 2930, 2931, 2932, 2933, 2934, 2935, 2936, 2937, 2938, 2939, 2940, 2941, 2942, 2943, 2944, 2945, 2946, 2947, 2948, 2949, 2950, 2951, 2952, 2953, 2954, 2955, 2956, 2957, 2958, 2959, 2960, 2961, 2962, 2963, 2964, 2965, 2966, 2967, 2968, 2969, 2970, 2971, 2972, 2973, 2974, 2975, 2976, 2977, 2978, 2979, 2980, 2981, 2982, 2983, 2984, 2985, 2986, 2987, 2988, 2989, 2990, 2991, 2992, 2993, 2994, 2995, 2996, 2997, 2998, 2999, 3000, 3001, 3002, 3003, 3004, 3005, 3006, 3007, 3008, 3009, 3010, 3011, 3012, 3013, 3014, 3015, 3016, 3017, 3018, 3019, 3020, 3021, 3022, 3023, 3024, 3025, 3026, 3027, 3028, 3029, 3030, 3031, 3032, 3033, 3034, 3035, 3036, 3037, 3038, 3039, 3040, 3041, 3042, 3043, 3044, 3045, 3046, 3047, 3048, 3049, 3050, 3051, 3052, 3053, 3054, 3055, 3056, 3057, 3058, 3059, 3060, 3061, 3062, 3063, 3064, 3065, 3066, 3067, 3068, 3069, 3070, 3071, 3072, 3073, 3074, 3075, 3076, 3077, 3078, 3079, 3080, 3081, 3082, 3083, 3084, 3085, 3086, 3087, 3088, 3089, 3090, 3091, 3092, 3093, 3094, 3095, 3096, 3097, 3098, 3099, 3100, 3101, 3102, 3103, 3104, 3105, 3106, 3107, 3108, 3109, 3110, 3111, 3112, 3113, 3114, 3115, 3116, 3117, 3118, 3119, 3120, 3121, 3122, 3123, 3124, 3125, 3126, 3127, 3128, 3129, 3130, 3131, 3132, 3133, 3134, 3135, 3136, 3137, 3138, 3139, 3140, 3141, 3142, 3143, 3144, 3145, 3146, 3147, 3148, 3149, 3150, 3151, 3152, 3153, 3154, 3155, 3156, 3157, 3158, 3159, 3160, 3161, 3162, 3163, 3164, 3165, 3166, 3167, 3168, 3169, 3170, 3171, 3172, 3173, 3174, 3175, 3176, 3177, 3178, 3179, 3180, 3181, 3182, 3183, 3184, 3185, 3186, 3187, 3188, 3189, 3190, 3191, 3192, 3193, 3194, 3195, 3196, 3197, 3198, 3199, 3200, 3201, 3202, 3203, 3204, 3205, 3206, 3207, 3208, 3209, 3210, 3211, 3212, 3213, 3214, 3215, 3216,

3217, 3218, 3219, 3220, 3221, 3222, 3223, 3224, 3225, 3226, 3227, 3228, 3229, 3230, 3231, 3232, 3233, 3234, 3235, 3236, 3237, 3238, 3239, 3240, 3241, 3242, 3243, 3244, 3245, 3246, 3247, 3248, 3249, 3250, 3251, 3252, 3253, 3254, 3255, 3256, 3257, 3258, 3259, 3260, 3261, 3262, 3263, 3264, 3265, 3266, 3267, 3268, 3269, 3270, 3271, 3272, 3273, 3274, 3275, 3276, 3277, 3278, 3279, 3280, 3281, 3282, 3283, 3284, 3285, 3286, 3287, 3288, 3289, 3290, 3291, 3292, 3293, 3294, 3295, 3296, 3297, 3298, 3299, 3300, 3301, 3302, 3303, 3304, 3305, 3306, 3307, 3308, 3309, 3310, 3311, 3312, 3313, 3314, 3315, 3316, 3317, 3318, 3319, 3320, 3321, 3322, 3323, 3324, 3325, 3326, 3327, 3328, 3329, 3330, 3331, 3332, 3333, 3334, 3335, 3336, 3337, 3338, 3339, 3340, 3341, 3342, 3343, 3344, 3345, 3346, 3347, 3348, 3349, 3350, 3351, 3352, 3353, 3354, 3355, 3356, 3357, 3358, 3359, 3360, 3361, 3362, 3363, 3364, 3365, 3366, 3367, 3368, 3369, 3370, 3371, 3372, 3373, 3374, 3375, 3376, 3377, 3378, 3379, 3380, 3381, 3382, 3383, 3384, 3385, 3386, 3387, 3388, 3389, 3390, 3391, 3392, 3393, 3394, 3395, 3396, 3397, 3398, 3399, 3400, 3401, 3402, 3403, 3404, 3405, 3406, 3407, 3408, 3409, 3410, 3411, 3412, 3413, 3414, 3415, 3416, 3417, 3418, 3419, 3420, 3421, 3422, 3423, 3424, 3425, 3426, 3427, 3428, 3429, 3430, 3431, 3432, 3433, 3434, 3435, 3436, 3437, 3438, 3439, 3440, 3441, 3442, 3443, 3444, 3445, 3446, 3447, 3448, 3449, 3450, 3451, 3452, 3453, 3454, 3455, 3456, 3457, 3458, 3459, 3460, 3461, 3462, 3463, 3464, 3465, 3466, 3467, 3468, 3469, 3470, 3471, 3472, 3473, 3474, 3475, 3476, 3477, 3478, 3479, 3480, 3481, 3482, 3483, 3484, 3485, 3486, 3487, 3488, 3489, 3490, 3491, 3492, 3493, 3494, 3495, 3496, 3497, 3498, 3499, 3500, 3501, 3502, 3503, 3504, 3505, 3506, 3507, 3508, 3509, 3510, 3511, 3512, 3513, 3514, 3515, 3516, 3517, 3518, 3519, 3520, 3521, 3522,

3523, 3524, 3525, 3526, 3527, 3528, 3529, 3530, 3531, 3532, 3533, 3534, 3535, 3536, 3537, 3538, 3539, 3540, 3541, 3542, 3543, 3544, 3545, 3546, 3547, 3548, 3549, 3550, 3551, 3552, 3553, 3554, 3555, 3556, 3557, 3558, 3559, 3560, 3561, 3562, 3563, 3564, 3565, 3566, 3567, 3568, 3569, 3570, 3571, 3572, 3573, 3574, 3575, 3576, 3577, 3578, 3579, 3580, 3581, 3582, 3583, 3584, 3585, 3586, 3587, 3588, 3589, 3590, 3591, 3592, 3593, 3594, 3595, 3596, 3597, 3598, 3599, 3600, 3601, 3602, 3603, 3604, 3605, 3606, 3607, 3608, 3609, 3610, 3611, 3612, 3613, 3614, 3615, 3616, 3617, 3618, 3619, 3620, 3621, 3622, 3623, 3624, 3625, 3626, 3627, 3628, 3629, 3630, 3631, 3632, 3633, 3634, 3635, 3636, 3637, 3638, 3639, 3640, 3641, 3642, 3643, 3644, 3645, 3646, 3647, 3648, 3649, 3650, 3651, 3652, 3653, 3654, 3655, 3656, 3657, 3658, 3659, 3660, 3661, 3662, 3663, 3664, 3665, 3666, 3667, 3668, 3669, 3670, 3671, 3672, 3673, 3674, 3675, 3676, 3677, 3678, 3679, 3680, 3681, 3682, 3683, 3684, 3685, 3686, 3687, 3688, 3689, 3690, 3691, 3692, 3693, 3694, 3695, 3696, 3697, 3698, 3699, 3700, 3701, 3702, 3703, 3704, 3705, 3706, 3707, 3708, 3709, 3710, 3711, 3712, 3713, 3714, 3715, 3716, 3717, 3718, 3719, 3720, 3721, 3722, 3723, 3724, 3725, 3726, 3727, 3728, 3729, 3730, 3731, 3732, 3733, 3734, 3735, 3736, 3737, 3738, 3739, 3740, 3741, 3742, 3743, 3744, 3745, 3746, 3747, 3748, 3749, 3750, 3751, 3752, 3753, 3754, 3755, 3756, 3757, 3758, 3759, 3760, 3761, 3762, 3763, 3764, 3765, 3766, 3767, 3768, 3769, 3770, 3771, 3772, 3773, 3774, 3775, 3776, 3777, 3778, 3779, 3780, 3781, 3782, 3783, 3784, 3785, 3786, 3787, 3788, 3789, 3790, 3791, 3792, 3793, 3794, 3795, 3796, 3797, 3798, 3799, 3800, 3801, 3802, 3803, 3804, 3805, 3806, 3807, 3808, 3809, 3810, 3811, 3812, 3813, 3814, 3815, 3816, 3817, 3818, 3819, 3820, 3821, 3822, 3823, 3824, 3825, 3826, 3827, 3828,

3829, 3830, 3831, 3832, 3833, 3834, 3835, 3836, 3837, 3838, 3839, 3840, 3841, 3842, 3843, 3844, 3845, 3846, 3847, 3848, 3849, 3850, 3851, 3852, 3853, 3854, 3855, 3856, 3857, 3858, 3859, 3860, 3861, 3862, 3863, 3864, 3865, 3866, 3867, 3868, 3869, 3870, 3871, 3872, 3873, 3874, 3875, 3876, 3877, 3878, 3879, 3880, 3881, 3882, 3883, 3884, 3885, 3886, 3887, 3888, 3889, 3890, 3891, 3892, 3893, 3894, 3895, 3896, 3897, 3898, 3899, 3900, 3901, 3902, 3903, 3904, 3905, 3906, 3907, 3908, 3909, 3910, 3911, 3912, 3913, 3914, 3915, 3916, 3917, 3918, 3919, 3920, 3921, 3922, 3923, 3924, 3925, 3926, 3927, 3928, 3929, 3930, 3931, 3932, 3933, 3934, 3935, 3936, 3937, 3938, 3939, 3940, 3941, 3942, 3943, 3944, 3945, 3946, 3947, 3948, 3949, 3950, 3951, 3952, 3953, 3954, 3955, 3956, 3957, 3958, 3959, 3960, 3961, 3962, 3963, 3964, 3965, 3966, 3967, 3968, 3969, 3970, 3971, 3972, 3973, 3974, 3975, 3976, 3977, 3978, 3979, 3980, 3981, 3982 or 3983 in any one of SEQ ID No.s 10, 12 or 14.

For example, SEQ ID No.1 represents the double stranded siRNA molecule, of strand length 21 nucleotides, having an antisense strand based on (and including) nucleotides 643-663 of SEQ ID No.10. SEQ ID No.2 represents the double stranded siRNA molecule having an antisense strand based on nucleotides 1903-1923 of SEQ ID No.10. SEQ ID No.3 represents the double stranded siRNA molecule having an antisense strand based on nucleotides 2399-2417 of SEQ ID No.10. SEQ ID No.4 represents the double stranded siRNA molecule having an antisense strand based on nucleotides 1277-1296 of SEQ ID No.10.

Accordingly, it is possible to prepare and utilise a wide variety of double stranded siRNA molecules, and each of these, together with their use in therapeutic methods for

the treatment of respiratory tract allergic and/or non-allergic disease form part of the present invention.

Sense and antisense strands

In this specification, with respect to double stranded siRNA molecules, the following definitions apply to the terminology "sense" and "antisense".

A given double stranded siRNA molecule typically comprises two strands of RNA, each one being substantially complementary in sequence to the other such that they can bind together to form a duplex by Watson/Crick base pairing. The siRNA molecules of the invention may be designed for the purpose of enabling one strand of the duplex to bind to a target ribonucleic acid. In this specification the target ribonucleic acid is usually a STAT6 mRNA.

One strand of the siRNA duplex has substantial sequence complementarity (i.e. usually at least 70% complementarity) to a contiguous sequence of nucleotides forming part of the RNA sequence of the target mRNA. This strand of the siRNA duplex is designated the antisense strand and is complementary, or substantially complementary, to a part of the target mRNA to which the antisense strand is intended to bind as part of the mechanism of action of the siRNA.

The other strand of the duplex siRNA corresponds to and has substantial sequence identity (i.e. usually at least 70% identity) to a contiguous sequence of nucleotides forming part of the target mRNA sequence. This is designated the "sense" strand of the siRNA duplex.

Repression and silencing

Ribonucleic acids of the invention are designed to repress or silence the expression of a target ribonucleic acid, peptide, polypeptide or protein or to repress a function of such ribonucleic acid, peptide, polypeptide or protein.

A repression of expression results in a decrease in the quantity of the target, preferably of a target protein, e.g. STAT6. For example, in a given cell the repression of a target (e.g. STAT6 protein) by administration of a ribonucleic acid of the invention results in a decrease in the quantity of the target relative to an untreated cell.

Repression of a function may be the decrease in transcription of an mRNA, or translation of a peptide or polypeptide.

Repression may be partial. Preferred degrees of repression are at least 50%, more preferably one of at least 60, 70, 80, 85 or 90%. A level of repression between 90% and 100% is considered a 'silencing' of expression or function.

Sequence identity

Percentage (%) sequence identity is defined as the percentage of nucleic acid residues in a candidate sequence that are identical with residues in the given listed sequence (referred to by the SEQ ID No.) after aligning the sequences and introducing gaps if necessary,

to achieve the maximum sequence identity. Sequence identity is preferably calculated over the entire length of the respective sequences.

Unless specified otherwise, where the aligned sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence. For example, where a given sequence comprises 100 nucleotides and the candidate sequence comprises 10 nucleotides, the candidate sequence can only have a maximum identity of 10% to the entire length of the given sequence. This is further illustrated in the following examples:

(A)

Given seq:	XXXXXXXXXXXXXXX	(15 nucleotides)
Comparison seq:	XXXXXYYYYYYY	(12 nucleotides)

% sequence identity = the number of identically matching nucleotides after alignment divided by the total number of nucleotides in the given sequence, i.e. (5 divided by 15) x 100 = 33.3%

(B)

Given seq:	XXXXXXXXXX	(10 nucleotides)
Comparison seq:	XXXXXYYYYYYZZYZ	(15 nucleotides)

% sequence identity = number of identical nucleotides after alignment divided by total number of nucleotides in the given sequence, i.e. (5 divided by 10) x 100 = 50%.

Alignment for purposes of determining percent nucleotide sequence identity can be achieved in various ways that are within the skill in the art.

Hybridisation stringency

In accordance with the present invention, nucleic acids having an appropriate level of sequence identity may be identified by using hybridisation and washing conditions of appropriate stringency.

For example, RNA-RNA hybridisations may be performed according to hybridisation methods well known to a person of skill in the art, e.g. the method of Sambrook et al., ("Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001).

Calculation of the melting temperature (T_m) at a given salt concentration is one method of determining hybridisation stringency. Nucleic acid duplexes of low sequence identity will have a lower T_m than nucleic acid duplexes of higher sequence identity.

One of the most accurate derivations of the melting temperature is the nearest-neighbour method. This method is well known to persons of skill in the art, is suitable for calculating the T_m of short nucleic acids and takes into account the actual sequence of the oligonucleotides as well as salt concentration and nucleic acid concentration.

The nearest-neighbour equation for both DNA and RNA based oligonucleotides is:

$$T_m = \left[\frac{[1000\Delta H^\circ]}{A + \Delta S^\circ + R\ln(C_t/4)}\right] - 273.15 + 16.6\log[Na^+]$$

where $\Delta H°$ (Kcal/mol) is the sum of the nearest-neighbour enthalpy changes for hybrids, A is a constant (-10.8) correcting for helix initiation, $\Delta S°$ is the sum of the nearest neighbour entropy changes, R is the Gas Constant (1.99 cal $K^{-1}mol^{-1}$) and C_t is the molar concentration of the oligonucleotide. $\Delta H°$ and $\Delta S°$ values for both DNA and RNA nearest neighbour bases are publicly available (e.g. from Genosys Biotechnologies Inc.).

In general for RNA-RNA hybridisations under very high stringency conditions, the melting temperature of RNA duplexes of 100% sequence identity would be expected to be approximately greater than or equal to 60°C, although the actual T_m for any given duplex requires empirical calculation.

Accordingly, nucleotide sequences can be categorised by an ability to hybridise under different hybridisation and washing stringency conditions which can be appropriately selected using the above equation or by other similar methods known to persons skilled in the art.

Sequences exhibiting 95-100% sequence identity are considered to hybridise under very high stringency conditions, sequences exhibiting 85-95% identity are considered to hybridise under high stringency conditions, sequences exhibiting 70-85% identity are considered to hybridise under intermediate stringency conditions, sequences exhibiting 60-70% identity are considered to hybridise under low stringency conditions and sequences exhibiting 50-60% identity are considered to hybridise under very low stringency conditions.

<u>STAT6</u>

In this specification, STAT6 may refer to any STAT6 nucleic acid, polypeptide, or to any homologue, mutant, derivative or fragment thereof.

In this specification, a STAT6 polypeptide or protein may be any peptide, polypeptide or protein having an amino acid sequence having a specified degree of sequence identity to one of SEQ ID No.s 9, 11 or 13 or to a fragment of one of SEQ ID No.s 9, 11 or 13 or to the peptide or polypeptide encoded by the nucleotide sequence of one of SEQ ID No.s 10, 12 or 14 or a fragment of one of SEQ ID No.s 10, 12 or 14. The specified degree of sequence identity may be from at least 60% to 100% sequence identity. More preferably, the specified degree of sequence identity may be one of at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity.

In this specification, a STAT6 nucleic acid may be any nucleic acid (DNA or RNA) having a nucleotide sequence having a specified degree of sequence identity to one of SEQ ID No.s 10, 12 or 14, to an RNA transcript of any one of these sequences, to a fragment of any one of the preceding sequences or to the complementary sequence of any one of these sequences or fragments. Alternatively a STAT6 nucleic acid may be one that hybridises to one of these sequence under high or very high stringency conditions. The specified degree of sequence identity may be from at least 60% to 100% sequence identity. More preferably, the specified degree of sequence identity may be one of at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity.

The human STAT6 polypeptide and nucleotide sequence is available from the NCBI (http://www.ncbi.nlm.nih.gov/) database under accession number NM_003153 (GI:23397677) (also see Figure 4).

The mouse STAT6 polypeptide and nucleotide sequence is available from the NCBI (http://www.ncbi.nlm.nih.gov/) database under accession number NM_009284 (GI:6678154) (also see Figure 5).

The rat STAT6 polypeptide and nucleotide sequence is available from the NCBI (http://www.ncbi.nlm.nih.gov/) database under accession number XM_343223 (GI:34865760) (also see Figure 6).

A STAT6 nucleic acid may preferably refer to the nucleic acid encoding a human STAT6 polypeptide or protein or a homologue thereof.

Alternatively, STAT6 may refer to nucleic acid encoding a non-human STAT6 polypeptide or homologue thereof. A non-human STAT6 may preferably be selected from any one of a rat, mouse or other rodent (including any animal in the order Rodentia), and may also be selected from a pig, sheep, non-human primate or other non-human vertebrate organism or non-human mammal.

STAT6 homologues preferably have at least 60% sequence identity to the STAT6 sequence of the given organism. More preferably the level of sequence identity is at least 70, 80, 90 or 95%.

Fragments

A fragment may comprise a nucleotide or amino acid sequence encoding a portion of the corresponding full length sequence. In this specification the corresponding full length sequence may be one of SEQ ID No.s 9 to 14. Said portion may be of defined length and may have a defined minimum and/or maximum length.

Accordingly, the fragment may comprise at least, i.e. have a minimum length of, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% of the corresponding full length sequence. The fragment may have a maximum length, i.e. be no longer than, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% of the corresponding full length sequence.

The fragment may comprise at least, i.e. have a minimum length of, 10 nucleotides or amino acids, more preferably at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000 nucleotides or amino acids.

The fragment may have a maximum length of, i.e. be no longer than, 10 nucleotides or amino acids, more preferably no longer than 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 150, 200, 300, 400, 500, 600, 700,

800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000 nucleotides or amino acids.

The fragment may have a length anywhere between the said minimum and maximum length.

The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Brief Description of the Figures

Figure 1 *Design of siRNA targeting STAT6.*
Targeted DNA sequences (SEQ ID No.s 15-18) encoding parts of the STAT6 mRNA and the corresponding duplex structure of the prepared siRNA (SEQ ID No.s 1-4) are shown.

Figure 2 *Inhibition of STAT6 Expression by RNAi.*

A549 cells were treated with individual siRNA at a final concentration of 100 nM.

(A). 60 hours after treatment cellular proteins were harvested, equal amounts (10 µg) separated by molecular weight using electrophoresis and immobilised onto synthetic membranes (Western Blotting). The presence of STAT6 protein (120 kDa) was then detected using a polyclonal anti-STAT6 antibody (Santa Cruz Biotechnology, Ca., USA) where the amount of STAT6 expression in each sample correlates with band density. Cells treated with STAT6(1) siRNA (SEQ ID No.1) had no detectable expression of STAT6 protein (no visible band). In lanes 2 & 3 (STAT6 (2) (SEQ ID No.2)-, STAT6(3) (SEQ ID No.3)-treated) STAT6 protein bands are barely detectable, indicating significant inhibition (>95%). STAT6(4) siRNA (SEQ ID No.4) was the least efficient although this siRNA still inhibited STAT6 expression by 90%. In contrast, control scrambled siRNA (scGAPDH, lane 6) had no effect on STAT6 expression. Similarly, STAT6 siRNA had no effect on GAPDH expression which is readily detectable as a 37 kDa protein band (using a GAPDH-specific antibody).

(B). STAT6 gene expression (mRNA production) in siRNA-treated cells was measured by real-time RT-PCR, allowing absolute quantification of gene expression. By comparing the amount of STAT6 expression to the housekeeping gene GAPDH (i.e. the ratio of STAT6/GAPDH expression: y-axis) the specific effects of siRNA can be measured. As shown, STAT6 siRNA (1-4) inhibit STAT6 mRNA by ≥ 90%. In contrast, cells treated with scGAPDH siRNA do not exhibit any reduction in STAT6 mRNA expression, indicating that the transfection procedure itself does not inhibit the STAT6 gene.

Figure 3 *RNAi of STAT6 leads to loss of STAT6 function.* To measure STAT6 activity A549 cells were cultured in the presence (right histogram) or absence (left histogram) of IL-4 (1 ng/ml) for 30 minutes prior to staining with anti-phospho-STAT6: Alexa fluor-647 labelled antibody (BD PharMingen, Oxford, UK). This antibody only recognises STAT6 molecules that are phosphorylated on tyrosine residue 641. After staining procedures, fluorescence in cells was measured by flow cytometry. In the histograms the amount of bound antibody is indicated by the relative amount of detectable fluorescence in individual cells (x-axis). The amount of fluorescence that is detectable above background levels is indicated in the gated region marked P2. As shown, IL-4 was capable of activating STAT6 in cells as indicated by the increase in fluorescence (top row, 35.3% versus 5.1% background in unstimulated cells). In contrast, when cells were treated with STAT6-specific siRNA, the ability of IL-4 to activate STAT6 was completely abolished (bottom row, 11.2% fluorescence in both stimulated and unstimulated cells)

Figure 4. Extract from accession number NM003153 [gi:23397677] in the NCBI database (http://www.ncbi.nlm.nih.gov/) showing amino acid sequence (SEQ ID No.9) and nucleotide sequence (SEQ ID No.10) for human STAT6.

Figure 5. Extract from accession number NM009284 [gi:6678154] in the NCBI database (http://www.ncbi.nlm.nih.gov/) showing amino acid sequence (SEQ ID No.11) and nucleotide sequence (SEQ ID No.12) for mouse STAT6.

Figure 6. Extract from accession number XM343223 [gi:34865760] in the NCBI database (http://www.ncbi.nlm.nih.gov/) showing amino acid sequence (SEQ ID No.13) and nucleotide sequence (SEQ ID No.14) for rat STAT6.

Figure 7. Inhibition of STAT6 expression in human lung epithelial cells by RNAi persists for several days. (G=GAPDH siRNA, J=Jurkatt cell lysate, M=size markers, all lanes=10 µg of protein, blots are representative of at least 3 individual experiments).

Figure 8. STAT6 siRNA are efficient at concentrations as low as 10nM. (G=GAPDH siRNA, bA=b-actin siRNA, J=Jurkatt cell lysate, M=size markers, all lanes=10 µg of protein, results representative of 3 independent experiments).

Figure 9. RNAi abolishes expression of functional STAT6 protein without inducing an interferon response.

Figure 10. STAT6 suppression by RNAi is readily achievable in diverse human lung cell types.

Figure 11. RNAi of STAT6 abolishes the ability of IL-4 to up-regulate eotaxin-1 mRNA expression in human lung epithelial cells.

Detailed Description of the Best Mode of the Invention

Specific details of the best mode contemplated by the inventors for carrying out the invention are set forth below, by way of example. It will be apparent to one skilled in the art that the present invention may be practiced without limitation to these specific details.

STAT6 siRNA

Sequences within the coding region of STAT6 mRNA (GenBank, U16031) only were selected for targeting by siRNA. Regions near the start codon (within 75 bases) were avoided as they may contain regulatory protein binding sites.

To ensure specificity, selected target sites were compared by BLAST® (NCBI) search for homology with other known coding sequences. Target sequences were also selected on the basis of having a GC content < 40% and beginning with AA to allow thymidine overhangs (tt) in the subsequent siRNA (Figure 1).

Pure STAT6 siRNA duplexes were chemically synthesised according to the inventors design by Ambion Inc. (Austin, TX, USA) and supplied as dried RNA oligonucleotide. Reconstituted siRNA were subsequently employed in cell treatment experiments.

Targeting of STAT6 Gene Expression by STAT6 siRNA

STAT6-expressing lung epithelial cells (A549) were treated with individual STAT6 siRNA and their ability to subsequently inhibit STAT6 expression determined by measuring both STAT6 mRNA and STAT6 protein expression (Figure 2). To ensure efficient cellular targeting, siRNA were complexed with a commercially available cationic lipid reagent (Lipofectamine™, Invitrogen) and transfected into cells.

STAT6 siRNA were validated against commercially available GAPDH ('housekeeping' gene) siRNA and scrambled GAPDH siRNA with no known homology to human mRNA sequences (Ambion, Inc.) i.e. positive and negative controls respectively. In these experiments STAT6 siRNA duplexes were shown to inhibit the expression of both STAT6 mRNA and STAT6 protein expression in treated cells. Furthermore, this suppression was STAT6-specific in that the expression of non-related housekeeping genes such as GAPDH, were not affected.

STAT6 Function is Abolished by STAT6 siRNA Treatment

In order for RNAi to be a successful therapeutic it is essential that the targeting of genes leads to loss of protein function within treated cells. Therefore, in addition to measuring STAT6 expression (as shown above), we determined the effects of siRNA treatment on STAT6 activity within cells. As STAT6 protein has to become phosphorylated within cells in order for it to mediate its effects, we employed an assay that directly measures the amount of phosphorylated STAT6 within intact cells. This assay utilises an anti-phospho-STAT6 antibody (BD-PharMingen) that fluorescently labels cells expressing the phosphorylated STAT6 protein. The amount of detectable fluorescence in IL-4 treated cells (measured by flow cytometry) is directly related to the amount of phosphorylated STAT6 (Figure 3).

To activate STAT6, cells were stimulated with interleukin-4 (IL-4), a chemical messenger that is produced during allergic responses and naturally activates STAT6 in cells. Using this assay we were able

to unequivocally demonstrate that RNAi of STAT6 leads to elimination of STAT6 function (phosphorylation) in cells.

A 90% inhibition of gene expression does not necessarily correlate with complete loss of STAT6 protein expression and therefore by extrapolation, its function within cells. Accordingly, the antibody staining experiments (flow cytometry - Figure 3) were performed. These results show that STAT6 siRNA treatment leads to loss of STAT6 function. STAT6 protein expression was inhibited following siRNA treatment (as demonstrated by Western Blotting) and this deficiency appears to be absolute in that STAT6-phosphorylation in response to interleukin-4 stimulation could not be detected by flow cytometry.

Inhibition of STAT6 expression in human lung epithelial cells by RNAi persists for several days

Referring to the results set out in Figure 7. A549 cells were transfected with 100 nM STAT6 siRNA 1-4 (SEQ ID No.s 1-4) on day 0. Protein and RNA fractions were prepared from cells harvested on day 3, day 5 & day 7 post-transfection and analysed for STAT6 protein/ mRNA expression by Western blotting (Figure 7 - top panels) and real-time RT-PCR respectively. On day 3, STAT6 protein expression was completely abolished by STAT6 siRNA treatment (GAPDH levels were unaltered). In contrast, scrambled siRNA (SC) did not inhibit STAT6 (or GAPDH) protein expression. The same effect was discernable on day 5 and day 7. Some recovery was detectable on day 7 after siRNA-4 treatment (Figure 7 - left blot).

RT-PCR analysis using human STAT6-specific primers confirmed STAT6 inhibition (normalised fold change relative to treatment with SC siRNA, $\Delta\Delta$Ct), STAT6 mRNA expression was significantly suppressed at all time points post-treatment.

<u>STAT6 siRNA are efficient at concentrations as low as 10nM</u>

To test the efficacy of STAT6 RNAi, A549 lung epithelial cells were transfected with various concentrations of individual STAT6 siRNA 1-4 (SEQ ID No.s 1-4). The results are set out in Figure 8. Cells were harvested on day 3 post-transfection and protein and RNA fractions subject to analysis for STAT6 protein/ mRNA expression by Western blotting (Figure 8 - top panel) and real-time RT-PCR respectively.

STAT6 siRNA were shown to inhibit STAT6 expression at all concentrations tested, whereas scrambled (SC) siRNA had no effect on STAT6 expression (GAPDH levels were unaffected by either treatment). RT-PCR analysis showed 50 nM to be the most potent siRNA concentration in terms of STAT6 mRNA inhibition (fold change, $\Delta\Delta$Ct) and this was confirmed at the protein level by Western blotting. However, even with 10 nM siRNA (equivalent to 0.13 ng/ml of dsRNA) there was significant suppression of STAT6 expression.

STAT6 siRNA-1 (SEQ ID No.1) and STAT6 siRNA-3 (SEQ ID No.3) completely inhibited STAT6 protein expression at 10 nM. STAT6 siRNA-2 (SEQ ID No.2) and STAT6-4 (SEQ ID No.4) showed weaker inhibition at the 10nM concentration (faint bands are observable on the Western blot).

<u>RNAi abolishes expression of functional STAT6 protein without inducing an interferon response</u>

Human lung epithelial cells (A549) were transfected with either 20 nM STAT6 siRNA-1 (SEQ ID No.1) (3 right panels) or with 20 nM scrambled (SC) siRNA that is non-homologous with any known human gene (mRNA). 3 days post-transfection, cells were stimulated with 100 ng/ml of human rIL-4 for 60 minutes, then harvested, fixed and stained for intracellular expression of phosphorylated-STAT6 using Alexa-Fluor-488 conjugated anti-human phospho-STAT6 (BD PharMingen). As dsRNA (including some siRNA) have been reported to activate an interferon response in certain human cell types, cells were also stained for STAT1-phosphorylation (STAT1 is specifically phosphorylated by interferon-receptor signalling) using a phospho-STAT1-specific antibody (BD-PharMingen).

The results are set out in Figure 9. In the absence of IL-4 stimulation, SC-siRNA-treated cells did not exhibit any detectable STAT6 phosphorylation (Figure 9 - left panel) when compared with isotype control stained cells. In contrast, IL-4 was able to readily induce STAT6 phosphorylation (Alex-Fluor-488 fluorescence) in SC-siRNA treated cells. When cells were treated with STAT6-1 siRNA this ability of IL-4 to phosphorylate STAT6 was abolished as indicated by the absence of fluorescent staining - indicating an absence of functional STAT6 protein in STAT6 siRNA-1 (SEQ ID No.1) treated cells. Intracellular staining with anti-phospho-STAT1 showed a complete absence of STAT1-phosphorylation in siRNA-treated cells (SC or STAT6-1), indicating that interferon signalling was not induced by siRNA-treatment (this is in

agreement with inventors RT-PCR data showing that the interferon-response gene OAS-1 is not modulated upon siRNA-treatment).

To confirm interferon-responsiveness, parallel siRNA-treated epithelial cell cultures were stimulated with exogenous human rIFN-γ (10 ng/ml for 60 minutes). Under these conditions, STAT1-phosphorylation was readily detectable by specific antibody staining (Figure 9 - right panel).

The combined results from these studies show that functional STAT6 protein expression is readily and specifically abolished in epithelial cells by 20 nM STAT6 siRNA and that siRNA treatment does not induce detectable interferon responses in targeted cells. Data is representative of at least 5 independent experiments.

<u>STAT6 suppression by RNAi is readily achievable in diverse human lung cell types</u>

HL cells (lung fibroblasts) were transfected with various concentrations of STAT6 siRNA-1 (SEQ ID No.1) and cells harvested on day 3 post-transfection were analysed for STAT6 expression by Western blotting, real-time RT-PCR & flow cytometric analysis. The results are set out in Figure 10.

Similar to epithelial cells, STAT6 protein expression in fibroblasts was completely and specifically abolished by STAT6 siRNA-1 (SEQ ID No.1) treatment (GAPDH levels were unaltered). 100 nM, 50 nM & 10 nM STAT6 siRNA-1 (SEQ ID No.1) all inhibited STAT6 protein expression by day 3 post-transfection (Figure 10 - top panel). In contrast,

scrambled siRNA (SC) did not modulate STAT6 or GAPDH protein expression. RT-PCR analysis confirmed STAT6 inhibition in that relative to SC/ housekeeping controls ($\Delta\Delta$Ct), STAT6 mRNA expression was significantly suppressed at all siRNA concentrations.

In this cell type, 10 nM STAT6-1 siRNA mediated the greatest inhibition of STAT6 mRNA levels (normalised mean fold change in STAT6 mRNA expression relative to treatment with SC siRNA = -97). To further confirm knockdown, siRNA-treated cells were stimulated with 100 ng/ml of human rIL-4 for 60 minutes and STAT6-phosphorylation measured by flow cytometric analysis (lower-right panel). Using a phospho-STAT6-specific antibody (BD-PharMingen), STAT6-phosphorylation was shown to be completely abolished in STAT6 siRNA-1 (SEQ ID No.1) -treated/IL-4 stimulated cells at all concentrations of STAT6 siRNA-1 (SEQ ID No.1), indicating an absence of functional STAT6 protein in these cells. In contrast, SC siRNA (100 nM) did not inhibit the ability of IL-4 to phosphorylate STAT6 in HL cells (indicated by relative increase in fluorescence).

<u>RNAi of STAT6 abolishes the ability of IL-4 to up-regulate eotaxin-1 mRNA expression in human lung epithelial cells.</u>

To further confirm functional STAT6 blockade, the ability of IL-4 to drive the expression of a known STAT6-responsive gene (eotaxin-1) in STAT6 siRNA-treated cells was determined. The results are shown in Figure 11.

Lung epithelial cells (A549) were transfected with STAT6-1 siRNA (SEQ ID No.1) or scrambled SC siRNA (20 nM final

concentration) and 3 days post-transfection were treated with human rIL-4 (10, 50 ng/ml) for a further 12 hours. Total RNA was then extracted from harvested cells and STAT6/ eotaxin-1 mRNA expression determined by real-time RT-PCR.

As illustrated in Figure 11 (left panel), STAT6 mRNA expression was significantly inhibited by STAT6-1 siRNA (SEQ ID No.1) treatment (relative fold change, ΔΔCt). In contrast SC siRNA had no effect on relative levels of STAT6 mRNA. When eotaxin-1 mRNA transcript levels were determined in the same samples using human eotaxin-1 specific primers (Figure 11 - right panel), IL-4 was shown to up-regulate the relative levels of eotaxin-1 mRNA levels in SC-siRNA-treated cells (ΔΔCt relative to unstimulated cells). In contrast, IL-4 did not significantly modulate eotaxin-1 mRNA expression in STAT6-1 siRNA (SEQ ID No.1)-treated cells.

Although these findings are preliminary and require validation at the protein level, they indicate that IL-4 induced regulation of eotaxin-1 gene expression in human lung epithelial cells is STAT6-dependent and confirm independent findings in mice and humans that eotaxin-1 is a STAT6-regulated gene.

These findings also illustrate the utility of STAT6 RNAi as a tool for investigating human STAT6 function and, given the reported importance of the IL-4-STAT6-eotaxin axis in allergic asthma, indicate the potential therapeutic benefit of inhibiting this pathway in vivo.

<u>Discussion</u>

The results described show that by designing siRNA specific to STAT6 effective inhibition of STAT6 gene (mRNA) and protein expression can be achieved in cell types that are relevant to asthma. Importantly, the results also demonstrate that treatment of cells with STAT6 siRNA leads to the abolition of STAT6 function upon stimulation with physiological stimuli.

The results show that STAT6 directed siRNA are active in successfully repressing the cellular expression and activity of STAT6 at very low concentrations, e.g. down to 10 nM. Prior art antisense techniques, which proved unsuccessful, required much higher concentrations of antisense DNA, often up to 100 times higher. The efficacy of low concentrations of STAT6 directed siRNA demonstrated here provides a significant improvement over the prior art. In particular, therapeutic efficacy at such low concentration alleviates many of the problems of delivery of high concentrations of active agent which remain in the cell without undergoing degradation for sufficient time for them to take effect.

As STAT6 is known to be a central mediator of many of the dysregulated processes that take place in allergic disease of the respiratory tract, the targeting of this gene by this approach provides a route of unique therapy for diseases including asthma, rhinitis and non-allergic asthma where STAT6 has also been implicated.

Accordingly, a STAT6 siRNA based treatment for respiratory tract allergic disease is provided which, in the case of asthma or rhinitis, may operate by selectively down-regulating STAT6 expression,

ameliorating the allergic inflammation-inducing effects of STAT6 in patients.

References

1. Barnes,P.J. & Hansel,T.T. The need for new therapy. *New Drugs for Asthma, Allergy and COPD. Prog. Respir. Res. (Karger)* **31**, 2-5 (2001).

2. Izuhara,K., Shirakawa,T., Adra,C.N., Hamasaki,N. & Hopkin,J.M. Emerging therapeutic targets in allergy: IL-4Ra and Stat6. *Emerging Theraputic Targets* **3**, 381-389 (1999).

3. Caplen,N.J. A new approach to the inhibition of gene expression. *Trends Biotech.* **20**, 49-51 (2002).

4. Elbashir,S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-498 (2001).

5. Elbashir,S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-498 (2001).

6. Foster,P.S. STAT6: an intracellular target for the inhibition of allergic disease. *Clin. Exp. Allergy* **29**, 12-16 (1999).

7. Izuhara,K., Shirakawa,T., Adra,C.N., Hamasaki,N. & Hopkin,J.M. Emerging therapeutic targets in allergy: IL-4Ra and Stat6. *Emerging Theraputic Targets* **3**, 381-389 (1999).

8. Bertrand,J-R et al. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochemical and Biophysical Research Communications* 296 (2002) 1000-1004.

9. Bitko, V et al. Inhibition of respiratory viruses by nasally administered siRNA. *Nature Medicine* Vol 11 No.1 January 2005 50-55.

10. Soutschek, J et al. Therapeutic silencing of an endogenous gene by systematic administration of modified siRNAs. *Nature* Vol 432, 11 November 2004 173-178.

11. Sioud,M et al. Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochemical and Biophysical Research Communications* 312 (2003) 1220-1225.

12. Massaro,D et al. Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice. *Am J Physiol Lung Cell Mol Physiol* L1066-L1070, 2 July 2004.

13. Stolzenberger, S et al. Specific inhibition of interleukin-4-dependent Stat6 activation by an intracellularly delivered peptide. *Eur J Biochem* 268 4809—4814 (2001).

14. Popescu,F-D. New asthma drugs acting on gene expression. *J Cell Mol Med* Vol 7 No.4 2003 475-486.

15. WO 98/40478.

16. Danahay, H; Hill, S; Owen, C.E. (2000). The in vitro and in vivo pharmacology of antisense

oligonucleotides targeted to murine STAT6. Inflamm. Res. 49: 692-699.